IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Chang
Application No. 09/701,536
Filed: June 18, 2001
Confirmation No. 5492
For: NUCLEIC ACID VACCINES FOR PREVENTION OF FLAVIVIRUS INFECTION
Examiner: Jeffrey S. Parkin
Art Unit: 1648
Attorney Reference No. 6395-64907-01

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I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: MAIL STOP AMENDMENT, COMMISSIONER FOR PATENTS, P.O. BOX 1450, ALEXANDRIA, VA 22313-1450 on the date shown below.

Attorney
for Applicant(s) _____
Tanya M. Harding, Ph.D.

Date Mailed October 6, 2004

DECLARATION UNDER 37 C.F.R. § 1.131

I, Gwong-Jen J. Chang, hereby declare as follows:

1. I am the inventor of the subject matter described and claimed by United States Patent Application No. 09/701,536, referenced above ("the '536 application"). I am currently employed by The Government of the United States of America as represented by the Secretary of the Department of Health and Human Services, Centers for Disease Control and Prevention (the CDC), the assignee of the '536 application. I was employed by the CDC in Fort Collins, Colorado while developing the invention described and claimed in the referenced application.

2. I understand that claims pending in the present application have been rejected in view of United States Patent No. 6,258,788 to Schmaljohn ("Schmaljohn"). I understand that Schmaljohn has been cited as allegedly anticipating certain claims pending in the '536 application, or, in the alternative, as allegedly rendering the claimed embodiments obvious.

3. The effective filing date of Schmaljohn is presumed to be no earlier than November 20, 1997. The '536 application was filed on June 3, 1999, and claims priority to and benefit of United States Provisional Application No. 60/087,908, filed June 4, 1998. However, I invented the subject matter covered by the claims pending in the '536 application well prior to the November 20, 1997 effective filing date of Schmaljohn, when it became available as a reference.

4. Accompanying this Declaration as Exhibit A are copies of pages from my laboratory research notebook. These copies are true and accurate facsimile copies of the corresponding pages from my laboratory notebooks. All dates stated on these pages have been redacted.

5. All entries on the notebook pages of Exhibit A were made prior to November 20, 1997.

6. Accompanying this Declaration as Exhibit B is a photocopy of the Employee Invention Report ("EIR") I submitted to my employer the CDC, describing various aspects of the subject matter of the '536 application. This is a true and accurate copy of the EIR that I submitted to the CDC. All dates stated on these pages have been redacted.

7. The EIR was submitted prior to November 20, 1997.

8. The ideas and concepts demonstrated by Exhibit A arose from work conducted for the CDC in my laboratory in Fort Collins, Colorado. These ideas and concepts are embodied in the claims of the '536 application. Thus, conception and reduction to practice of the invention recited in the claims of the '536 application, as discussed in more detail below, occurred in the United States of America prior to November 20, 1997.

9. Exhibit A consists of 15 pages of laboratory notebook pages. The contents of these pages of Exhibits A, and pertinent statements made on these pages are discussed below.

A. Pages 1-7 of Exhibit A document the identification of a plasmid incorporating polynucleotide sequences encoding the prM and E proteins of Japanese Encephalitis Virus ("JEV"). These experiments are described in detail in Example 1 on pages 19-21 of the '536 application.

1) Page 1 describes the selection of several candidate colonies resulting from the cloning experiments inserting the prM and E protein coding sequences into a suitable plasmid expression vector.

2) Page 2 shows the results of restriction enzyme digestion and electrophoretic sizing of the candidate clones, illustrating that multiple clones contained an insert of the correct size to contain the prM and E DNA.

3) Pages 3 and 4 document the large scale purification of plasmids, including plasmid 2-7 selected as a vaccine.

4) All results documented on pages 1-4 of Exhibit A were completed before November 20, 1997.

B. Pages 5-6 of Exhibit A document the introduction (by transfection) of plasmids including the prM-E sequences into mammalian cells, and the characterization of the proteins expressed from the transfected plasmids by immunofluroescence assay ("IFA"). These experiments are described in detail in Example 2 (including Table 1), on pages 21-23 of the '536 application.

1) Page 5 describes the transfection of candidate plasmids into SVT2, COS-1 and COS-7 cells.

2) Page 6 documents the results of an IFA showing that cells expressing the 2-7 plasmid express the JEV antigen.

3) All results documented on pages 5-6 of Exhibit A were completed before November 20, 1997.

C. Pages 7-9 of Exhibit A document the construction of an alternative plasmid designated pCBJE1-14 designed to increase expression of the JEV sequences. Details of the construction and evaluation of the pCBJE1-14 plasmid vector are described in Examples 1 and 2, on pages 19-23 of the '536 application.

1) Page 7 schematically illustrates the elements of the plasmid backbone designed to give enhanced expression of JEV sequences incorporated into the vector.

2) Page 8 and 9 document insertion of the JEV DNA sequences into the vector backbone. Page 9 confirms that the pCBJE1-14 includes the correct JEV DNA sequences.

3) These results were obtained prior to November 20, 1997.

D. Page 10 of Exhibit A shows the characterization of the JEV E protein expressed from the of the JE-4B cell clone selected for recombinant antigen production as the biosynthetic subunit vaccine and serodiagnostic antigen. Characterization of the expressed E protein was performed using a panel of monoclonal antibodies specific for various epitopes of the JEV E protein. These results are described in detail in the text of Example 3 on page 24 and in Table 2 on page 25 of the '536 application. All results documented on page 7 of Exhibit A were completed before November 20, 1997.

E. Pages 11-14 of Exhibit A describe the preparation of, and immunization of mice with, the JEV DNA vaccine (pCDJE2-7). Example 5 on pages 27-29 details these experimental results. Page 8 illustrates the preparation of the DNA vaccine.

1) Page 11 and 12 outline the immunization protocol.

2) Page 13 documents assay of serum collected from mice immunized with the JEV DNA vaccine.

3) Page 14 describes the enzyme-linked immunosorbent assay ("ELISA") used to determine antibody production in the serum of immunized mice, and the raw data resulting from an ELISA showing the presence of antibodies specific for JEV in the serum of immunized mice.

4) These and similar results obtained from serum collected at subsequent time points from the same immunized mice are provided in Table 3, on page 29 of the '536 application. Mice were immunized, and serum collected at 3, 6, 9, 23, 40 and 60 weeks post-immunization.

5) All of these results were obtained prior to November 20, 1997.

F. Page 15 of Exhibit A documents experiments designed to evaluate the effectiveness of neonatal immunization with the JEV DNA vaccine. These experiments are

detailed in Examples 6 and 7 on pages 30-32 of the '536 application. These results demonstrated that the JEV DNA vaccine claimed in the '536 application was effective at protecting immunized animals against viral challenge. These results were obtained prior to November 20, 1997.

10. Exhibit B consists of a five page Employee Invention Report submitted by me to the CDC. The contents of Exhibits B, and pertinent statements made on the pages of Exhibit B are discussed below.

11. Page 3 of Exhibit B is a description of certain aspects of the subject matter which is the subject of the '536 application. This is a brief summary of experiments and results that demonstrated the production of an effective DNA vaccine for JEV. For example, I described the production of a long-lasting protective antibody response following immunization with the JEV DNA vaccine that is an embodiment of the invention claimed in the '536 application. The EIR provided as exhibit B was submitted to the CDC for review before November 20, 1997.

12. In conclusion, Exhibits A and B demonstrate that I invented the subject matter claimed in the '536 application before November 20, 1997, the date on which US Patent No. 6,258,788 to Schmaljohn became available as a reference.

13. All statements made herein and of my own knowledge are true and all statements made on information are believed to be true. Furthermore, these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements made may jeopardize the validity of the application or any patent issuing thereon.

_____ _____

Date Gwong-Jen J. Chang

Name:

Experiment:

* Transform XR2 & XR12 into Top10F' Cells

Plate out 090595 Clone 12, 27, 32 in LBAmp plates

{XR2 & XR12 gave Rame size colonies "0"
090595 Vector Control
But 090595-L had mixed size colonies a few colonies had same size as of Vecta control "0".
Most of the colonies were pin point in size "."

I pick one each from XR2 & XR12; 090595-12, 27, 32
(AH-12, 27, 32)
and 25 from new 090595-L plate (I pick one unique colony and label as "1")
* Inoculate into 4ml LB-Amp25 medium *
grow at 37°c, 280 rpm for 24 hrs)
(from 9.00 am —

Purification Results: 6ml of O/N culture in 160μl dH2O

XR2	25.5μg/ml	4μg
XR12	21.5μg/ml	3.44μg
AH12	443μg/ml	70μg
AH27	413μg/ml	66.08μg
AH32	380μg/ml	60.8μg

HpaI Digestion

XR2	9.8μl	XR12	11.6μl	XR2	9.8μl
10X4	2.0μl	10XNEB4	2.0μl	NEB2	2.0μl
HpaI	1.0μl	HpaI	1.0μl	NheI	1.0μl
dH2O	7.2μl		5.4μl	EcoRV	1.0μl
				dH2O	6.2μl

NheI/EcoRV

XR12	11.6μl		
	2.0μl		
NheI	1.0		
	1.0μl		
	4.4μl		

Name:

Experiment:

AH12	1.12 µl / 100 ng
10×NEB4	2.0 µl
HpaI	1.0 µl
dH2O	15.88 µl

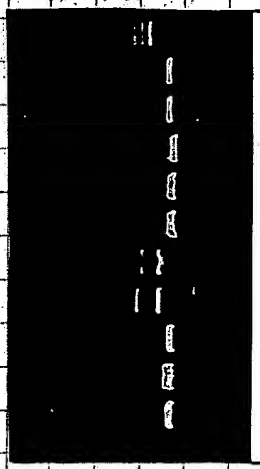
AH12	1.12 µl
10×NEB2	2.0 µl
EcoRV	1.0 µl
NheI	1.0 µl
dH2O	14.88 µl

AH27	1.21 µl
10×NEB4	2.0 µl
HpaI	1.0 µl
dH2O	15.79 µl

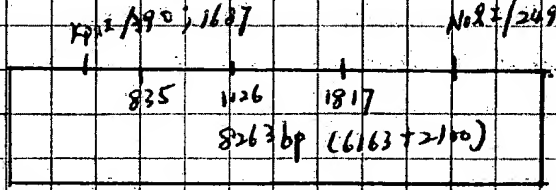
AH27	1.21 µl
10×NEB2	2.0 µl
EcoRV	1.0 µl
NheI	1.0 µl
dH2O	14.79 µl

AH32	1.31 µl
10×NEB4	2.0
HpaI	1.0
dH2O	15.69 µl

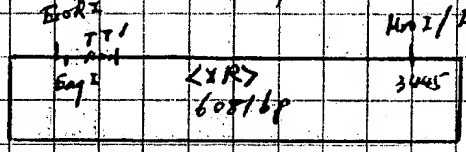
AH32	1.31 µl
10×NEB2	2.0 µl
EcoRV	1.0
NheI	1.0
dH2O	14.69 µl



Mis.
XR2/HpaI
XR22/HpaI
AH12/HpaI
AH27/HpaI
AH32/HpaI
XR2/EcoRV/NheI
XR12/EcoRV/NheI
AH12/ "
AH27/ "
AH32/ "



pCDNA3 = 6229 bp



EcoRV (825, 2767)
NheI (1126)
HpaI (1817)
XR/EcoRV/NheI size expected
(2918, 1641, 4149)
XR/HpaI (6081)

AH/EcoRV/NheI (991, 7972)
AH/HpaI (8263)

* I may have correct clone XR-12 for AH4 5'
* AH12 & AH32 for DNA Vaccine
⇒ Sequence AH12 & AH32

Name:

Experiment:

Grow up & Purification

Clone XR-12 for SA145' & GSF3,4,5,6 → in XL Blue MR
in TYP1F'
AH-12, AH32
#2-7, #2-3

Streak on LB Amp plate
Pick single colony & Grow in 5 ml LB Amp overnight

Inoculate into 1:100 LB Amp
200 ml for XR-12, & GS-F3456
AH-12, AH32, #2-3, #2-7

Purify

AH12-1	320 ng/μl	8263 bp	(1240 ng/SEC)
AH12-2	325 ng/μl		
#2-7-1	622 ng/μl		
#2-7-2	53.5 ng/μl		
#2-3-1	54 ng/μl		
#2-3-2	75 ng/μl		
AH32-1	320 ng/μl		
AH32-2	340 ng/μl		
XR12-1	68.5 ng/μl	6081 bp	(912 ng/SEC)
XR12-2	67 ng/μl		
GSF3456-1	90.5 ng/μl	10167 bp	(1525 ng/SEC)
GSF3456-2	94.5 ng/μl		

AH12-1	77.5 μl	ppt resuspend in 140 μl dH2O
AH32-1	77.5 μl	"
#2-3-2	330.0 μl	"
#2-7-1	400.0 μl	"
XR12-1	246.0 μl	"
GS-1	337.0 μl	"

[redacted]

pGH Control 18-9
#2-3-2 1μl
#AH32-1 1μl
XR12-1 1μl
#2-7-1 1μl
#AH32-1 1μl
GS-1 1μl

Important: Place card under blue copy.

Name:

Experiment:

Grow 500 ml of AH12, AH32, 2-3, 2-7 Purify by Qiagene Column

[redacted]

AH12 ml ≈ 1 pellet of Walter
AH32
2-3
2-7

[redacted]

AH12-1
AH12-2 1 ml ea
AH12-3
AH32-1
AH32-2
AH32-3
AH32-4
2-3-1
2-3-2
2-3-3
2-7-1
2-7-2
2-7-3

Name:

Experiment: DNA Vaccine

Recover AH12, 2-7, CE8, CE11 from Glycerol, & 14 SA14ZC
Streak out in LB Amp ⅓ ON at 37°C
Pick single colony from plate & inoculate in To 3 ml LB Amp
Grow cells at 30°C 22 hrs.
Inoculate 1:100 into 1 5ml LB Amp 100 µg/ml, 30°C, 22 hrs
Purify plasmid & resuspend in 1500 µl P1
1500 µl P2
Qiagen Tip 2100 µl N3
→ 300 ~ 400 µl TE or dH2O

AH12	TE
2-7	
CE8	
CE11	

SA14ZC-1	dH2O
2	5µl/300µl
3	
4	
6	
7	
11	
12	
13	
17	
18	
19	
21	
35	

Transform SVT2, COS1, COS7 cells with (
10 µl ea of AH12, 2-7, CE8, CE11
Electroporation condition on p 12
e⁻ once cell survival well
e⁻ twice 90% cell death b.c. COS1 > COS7 > SVT2

Passage e⁻ once cells from all constructs
1:4 passage in 25 cm² flasks
without Antibiotics
Next day add with 400 µg/ml (equal to 6 ml culture)
Geneticin 1 ml (G418
Antibiotic stock / G418 ; 20 mg/ml in PBS
Hygromycin B 1 ml/ml in PBS

Important: Place card under blue copy.

Name:

Experiment:

IFA with 1:50 dilution of mouse anti: SA14 serum

	SVT2	COS1	COS7
AH12	-/-	-/-	-/-
2-7	+/1%	++/30%	++/40%
CE8	++/2%	++/3%	++/4%
CE11	++/2%	++/2%	++/3%

∴ mutated DNA ?

(Store SVT2, COS & COS7 P4 Cells in N2 Cell NO 2 & Label SVT2 P4 Jc)

Select Stable Transformed Cell

G418 & Hygromycin B Concentration

G418 800 ug/ml (Label as 400)

HyB 400 ug/ml

2nd Change medium

Store

SVT2 2-7
SVT2 AH12 NO2
SVT2 CE8/ NO3
" CE8
COS1 AH12
COS1 2-7

Name:

Experiment:

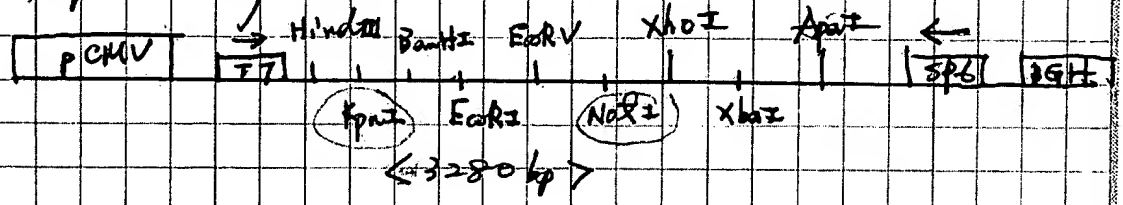
* I had completed Vector reconstruction. They are ready to receive Flavivirus Sequence.

Two vectors were constructed.

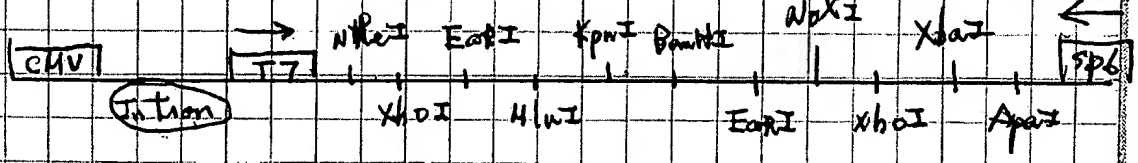
pCBamp is derived from pCDNA3, I removed f1 origin + SV40 origin/enhancer + Neomycin gene from nt. 4289 - 3455 of pUC deletion clone.

pCIB is derived from pCBamp1 & pCI (Promega) I replace NcoI (611nt) to KpnI (939nt) of pCBamp-1 with NcoI (5164) to KpnI (1080) of pCI.

pCBamp Cloning sites.



pCIBamp



Important: Place card under blue copy.

Name: D

Experiment: JE DNA Vaccine

Insert JE prM-E into pCBamp & pCIBamp Vector

RE Digestion:

JE prME in pCDNA3 Clone 2-7 620ng/λ

<KpnI>

2-7 (620ng/λ)	20μl/620ng
10× React 4	6μl
KpnI	3μl
dH_2O	33μl

37°C 1h, take 5μl after 45 min Run on 1% Gel to check the completion of digestion

5μl + 5μl dye

1μl uncut + 5μl " + 4μl dH_2O

Qiaquick PCR column Purified & resuspend in 80μl dH_2O

<NotI Digestion>

80μl 2-7 /KpnI	80μl
10× React 3	10μl
100× BSA	1μl
NotI	2μl
dH_2O	7μl

37°C for 1-2 hr.

Take 10μl after 1 hr, run on 1% Gel to check the completion of digestion

10μl + 1.1μl 10× loading dye

<Prepare 0.8% TAE purification Gel)

50 ml 1× TAE

0.4g Purification agarose

Run at 50 V for 40 min

Stain Gel by adding 5μl EtBr in 100μl dH_2O for 5 min

Important: Place card under blue copy.

Name:

Experiment:

Sequence Results

Clone 1-14, 1-15 in pCBamp have identical sequence
as 2-7 in pCDNA3 (DNA & sequence
1289
up to PvuI (at 3455 region) in pCDNA3)

* Complete construction of JE prM-E pCBamp construct
(done same pJECB1-14 & pJECB1-15

⇒ I need to reclone JE prM-E in To pCBamp
Vector.

1-8, 1-12, 2-2, 2-18 all have E. coli HSPs sequence
insert of 286 bp

Why ?

Sequences producing High-scoring Segment Pairs:

	High Score	Sum Probability P(N)	N
gb|L10328|ECOUW82 E. coli; the region from 81.5 to 84.5 m...	1430	2.9e-111	1

gb|L10328|ECOUW82 E. coli; the region from 81.5 to 84.5 minutes
Length = 136,254

Minus Strand HSPs:

Score = 1430 (395.1 bits), Expect = 2.9e-111, P = 2.9e-111
Identities = 286/286 (100%), Positives = 286/286 (100%), Strand = Minus / Plus

```
Query:    286 GAGTTCATTTATGGTTCGCTGCATTTATTTGACCCGATTATAAACACGGAATTTTCCCCG 227
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  20746 GAGTTCATTTATGGTTCGCTGCATTTATTTGACCCGATTATAAACACGGAATTTTCCCCG 20805

Query:    226 CAGGGCGTAGCGCTGCGCCAGTTCACCAGCCGCTGGGAAGGGGGTATGGTCAGAACGTCA 167
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  20806 CAGGGCGTAGCGCTGCGCCAGTTCACCAGCCGCTGGGAAGGGGGTATGGTCAGAACGTCA 20865

Query:    166 GGGAACTGGCTGCGTGACGGGAAAACGTTGATCCTTGATGATGCGGCAATTGCCGGGCTG 107
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  20866 GGGAACTGGCTGCGTGACGGGAAAACGTTGATCCTTGATGATGCGGCAATTGCCGGGCTG 20925

Query:    106 GAATATACCTTGCCGAAAAACTGGCAACAGTTGTGGATGGAAACGACACCCGGTTGGTTA 47
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  20926 GAATATACCTTGCCGAAAAACTGGCAACAGTTGTGGATGGAAACGACACCCGGTTGGTTA 20985

Query:     46 AACAGCCTGCAACTGAAGAGATTTAGCCCCAGCCGCAATCTGATCA 1
              ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  20986 AACAGCCTGCAACTGAAGAGATTTAGCCCCAGCCGCAATCTGATCA 21031
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SA14 Primary Sera
WEE HIAF

Antigen : JE 2-7 in COS/14B +
Antibodies : 1:50 in PBS

① G418 (-)	SA14IS	G3546	MC3	2F2	6B6C-1	WEEHIAF
	SA14IS	G3546	MC3	2F2	6B6C-1	WEE HIAF
② G418 (+)	SA14IS	G3546	MC3	2F2	6B6C-1	WEEHIAF
	SA14IC	G3546	MC3	2F2	6B6C-1	WEEHIAF
③ G418 +	JE301	JE109	JE112	JE203	JE204	JE201
	SA14IS	TC-83B4C-4 PBS		JE504	JE503	N.04
④ G418 +	JE301	JE109	JE112	JE203	JE204	JE201
	SA14IC	TC-83 3B4C-4 PBS		JE504	JE503	N.04

* Neutalizing Ab
◊ HI

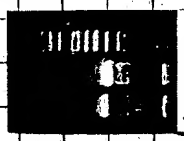
Name:

Experiment:

Preparation to test JE DNA Vaccine

DNA prep. 2-7)
pcDNA3CAT)

Gel
1μl + 9μl dye Run 100 V



OD_{260}
10μl + 90μl DEPC dH_2O 1.0 A_{260} = 60ng/ml

2-7 OD_{260} = 1.916 = 96ng/μl × 10 = 960ng/μl (original)

pcDNA3CAT OD_{260} = 0.458 = 23ng/μl × 10 = 230ng/μl (original)

2-7 : 2.420 ml Total = 2323.2μg + 6.05μl Salty Etoh
Resuspend in ~~2320μl dH2O~~ 1161.6μl dH_2O
< : 2μg/μl >

pcDNA3CAT : 5.035 ml Total = 1158.05μg + 2.5μl Salty Etoh
Resuspend in 290μl dH_2O each
< : 4μg/μl >

Name:

Experiment: Mouse Vaccination

Mouse NO. 1: 1R (mouse facing you, R is your Right)
2: 1L (mouse Right)
3: 2R
4: 2L
5: None

Group 1: 5 mice Received pcDNA3-CAT 0.5μg/1ml 100μl, 50μl each side of leg muscle (IM)

2 & 3: 5 mice each, received 2-7 DNA 1.0μg/1ml 100μl, 50μl each side of leg muscle (IM)

4: 5 mice received 1/5 (200μl) Human dose of Biken vaccine (S.C.)

* JE 2-7 1μg/1ml
pcDNA3CAT 0.5μg/1ml "

[redacted]

JAPANESE ENCEPHALITIS VACCINE
LYOPHILIZED
"BIKEN"

LYOPHILIZED JAPANESE ENCEPHALITIS VACCINE "BIKEN" which has been developed by the Research Foundation for Microbial Diseases of Osaka University, Suita, Osaka, Japan, provides active immunization against Japanese encephalitis (JE).

METHOD OF MANUFACTURE

Mice are inoculated intracerebrally with JE virus, "Nakayama-NIH" strain. After their full development of illness, brains are harvested and homogenized in phosphate-buffered saline, pH 8.0. The homogenate is centrifuged at low speed, and the supernatant is treated with protamine sulfate and then inactivated with formalin at lowered temperature. The inactivated virus suspension is purified by physico-chemical methods. Finally it is applied, on a sucrose cushion, centrifugation at 59,000 × g for 13 hours. The supernatant is slowly removed until 1/6 volume of the bottom layer is left. The pellet and bottom 1/6 portion of the supernatant are homogenized and diluted in 3.7 times concentrated TC medium 199 containing 0.175% gelatin and phosphate buffer, pH 7.2, together with a stabilizer for lyophilization to yield a 3.7 times concentrated suspension as to the final reconstituted vaccine. Of the suspension, 0.35 ml is lyophilized in a final container and sealed under dry pure nitrogen atmosphere.

RECONSTITUTION

The vial contains single dose of vaccine. For reconstitution, remove center tab of flip off cap. DO NOT REMOVE RUBBER STOPPER. Cleanse the stopper with tincture of iodine or 70% ethanol. The syringe and needle must be sterilized by autoclaving or boiling. Withdraw 1.3 ml of the sterile distilled water into the syringe. Insert needle into vial through center of stopper and introduce the diluent into the vial. Withdraw the air (nitrogen) into the syringe before drawing needle away from vial. Shake the vial thoroughly. The reconstituted vaccine should be used as soon as possible without any storage to avoid contamination as the vaccine contains only decreased amount of preservative after restoration. DO NOT FREEZE THE RECONSTITUTED VACCINE.

ADMINISTRATION

For initial immunization, usually two doses of 1 ml each are administered subcutaneously

Important: Place card under blue copy

Name:

Experiment: JE DNA vaccine

Collect mouse serum from vaccination
Nicotainer labeled as
2-7 (A) 1~5 ; 3 wks PV-1 ;
Biken 1~5 ; "
CAT 1~5 ; "
2-7 (B) 1~5 ; "

Test antibody titer by ELISA

Antigen: Purified SA14 50 ng/well
Serum: Test Serum
: Positive Serum SA14 Immune Serum
Dilution 1:100 ; 1:400 ; 1:1600

Results:

	ODnm at 1:100	End point Titer
2-7 A-1	1.765	>1,600
2-7 A-2	1.000	>1,600 400
2-7 A-3	0.864	400
2-7 A-4	1.764	400 >1600
2-7 A-5	1.070	>1,600
2-7 B-1	2.145	>1,600
2-7 B-2	0.871	400
2-7 B-3	0.693	100
2-7 B-4	1.348	400
2-7 B-5	0.660	400
Biken 1	0.978	>1,600
Biken 2	0.748	400
Biken 3	1.053	400
Biken 4	2.475	>1,600
Biken 5	2.205	>1600
CAT 1	0.184	<100
CAT 2	0.269	<100
CAT 3	0.169	<100
CAT 4	0.123	<100 X No amplification
CAT 5	0.188	<100
SA14 IS	2.149	>1,600
Naive HZAT	2.498	>1,600

Important: Place card under blue copy.

2x CAT Control = 0.34

Test:
Operator:
Batch name:

Units:
Date:

Read at 1:30pm

Biken 1 2-7A 2-7A 2-7A 2-7A 2-7A 2-7B 2-7B 2-7B 2-7B 2-7B Plate number
2 3 4 5 1 2 3 4 5

Row\Col	1	2	3	4	5	6	7	8	9	10	11	C
A	-0.009	-0.013	-0.005	-0.012	-0.004	-0.006	-0.006	-0.007	-0.008	-0.010	-0.009	-0
B	0.978	1.765	1.000	0.864	1.764	1.870	2.145	0.871	0.693	1.348	0.660	0
C	0.514	0.918	0.522	0.432	0.942	0.897	1.864	0.402	0.264	0.611	0.334	0
D	0.444	0.455	0.258	0.280	0.380	0.328	1.029	0.181	0.149	0.241	0.158	0
E	2.498	0.748	1.053	2.475	2.205	0.184	0.264	0.169	0.123	0.188	2.149	0
F	2.485	0.698	0.677	2.208	1.256	0.166	0.148	0.175	0.138	0.134	2.176	0
G	2.275	0.205	0.450	1.171	0.500	0.175	0.180	0.249	0.147	0.130	1.718	0
H	-0.012	-0.014	-0.013	-0.013	-0.014	-0.015	-0.014	-0.014	-0.013	-0.012	-0.011	-0

Naka Biken Biken Biken Biken CAT CAT CAT CAT CAT SA14
HIAF 2 3 4 5 1 2 3 4 5 1s

JE DNA Vaccination

① Coat plate 50ng/50µl SA14 Purified virus O/N at 4°
PBS Wash 5x

② Block 3% Goat serum in PBS 100µl/well
7:20 am – 8:00 am

③ Serum Dilution 1:100; 400x; 1600x
50µl/well 10:00 am – 11:00 am

④ HRP conjugated goat α-mouse Ig 25µl/well
mouse Ig 1:500 (120µl + 60.0ml) 11:10 am –

⑤ Substrate Sigma 104 phosphatase substrate
3 mg/ml 6mg/Tablet
1M Tris pH 8 12:30 pm

Name:

Experiment: JE DNA vaccine

Mouse Immunization

Mouse : 3 wks old, ♀, ICR mouse In 5 Group, 10 per Group.

3 days old, ICR mouse, 5 litter, 10 per litter.

DNA Construct: 2-7 (JE prM-E in pcDNA3) 1mg/ml
1-14 C " in pCBamp) 1 mg/ml
5-14 C " in pCIBamp) "

Biken Control : Nakayama/NIH 3 single human dose

Negative DNA Control : CAT in pcDNA3

Dosage; 3 wks old 100 μg/100 μl } for plasmid DNA
3 day old 50 μg/50 μl }
Biken; 3 wks old 1/5 Human dose 600 μl diluent
3 days old 1/100 Human dose

Collect serum from each mouse at (3 wks PV)
(7 wks PV)

1. Ear mark make each mice

1 = 1R 11 = 3L
2 = 1L Challenge with 3×10^8 pfu/100 μl IP
3 = 2R Original virus Titer = 6.3×10^9 pfu/ml
4 = 2L Diluted 6.3×10^3 fold = 10^6 pfu/10 μl
5 = 0
6 = 1R 1L IP : 100 μl IC : 30 μl BA-1
7 = 1R 2L
8 = 1L 2R
9 = 3R 2L
10 = 3R

Important: Place card under blue copy.

National Institute of Infectious Diseases

APPLICATION NO. 09/701,536

EXHIBIT B

PAGE 1

62202 /6314

CONFIDENTIAL

PHS Employee Invention Report

Use plain paper if more space is needed.

For Patent Branch Use
E-Number
U.S.P.A.#
U.S. Filing *(date)*

Part I: To Be Completed by the Inventor

First Inventor's Name: Chang, Gwong-Jen.J. **Phone No.** (970) 221-6497

1. **Give a short descriptive title of your discovery or invention.**
   Nucleic acid vaccines for the prevention of flavivirus infection.

2. **Please provide (in non-scientific terms if possible) a one paragraph description of the essence of your discovery or invention and identify the public health need it fills.**
   Please see the attached.

3. **Who contributed to the invention or discovery? Please identify all colleagues who *could* merit co-authorship credit for the associated publication, whether or not you believe them to be "co-inventors."**
   Chang, Gwong-Jen J. (inventor)
   Hunt, Ann (provided technical support of performing western blot, ELISA, HI and serological tests).
   Davis, Brent (provided technical support of performing large-scale plasmid purification and animal testing).

4. **Is anyone outside of the Public Health Service aware of your invention or discovery? If so, please identify them and describe the dates and circumstances.**
   No.

5. **Are you aware of any PHS patent applications that are related to your invention or discovery?**
   No.

6. **Please list the most pertinent previous articles, presentations or other public disclosures, made by you or by other researchers, that are related to your invention or discovery. Also, attach copies, *please!***
   Schalich, J., Allison, S.L., Stiasny, K., Mandl, C.W., Kunz, C., and Heinz, F.X. (1996). Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying envelope glycoprotein functions. J. Virol. 70:4549-57.

   See attachment for continuation.

7. Please indicate any future dates on which you will publish articles or make *any* presentations related to your invention or discovery.

We plan to submit the manuscript for publication and present the results in the spring of 1997.

8. In one paragraph, please speculate (and be creative!) about possible commercial uses of your invention or discovery.

Please see the attached.

9. a. Is the subject matter of your invention related to a PHS CRADA (Cooperative Research and Development Agreement) involving your laboratory or ICD?

☒ No
☐ Yes. If yes, please identify the collaborator: _____

b. Is the subject matter based on research materials that you obtained from some other laboratory?

☒ No
☐ Yes. If yes, please attach any material transfer agreements (MTA) under which you received the material.

10. What companies or academic research groups are conducting similar research (if you know)? Can you identify any companies that may be good licensing prospects?

Similar research has been conducted by the following institutes:
USAMRIID: tick-borne encephalitis virus
WRAIR: dengue viruses
USNMRC: dengue viruses
Chemical and Biological Defense Establishment, UK: St. Louis encephalitis virus.

11. What further research would be necessary for commercialization of your invention? Generally, what are your future research plans for the invention and/or for research in areas related to the invention?

We need to assess fully the risk of DNA integration and anti-dsDNA antibodies that may result in an increased risk of developing cancer or autoimmune disease in the vaccinated individual. After completion of the test in laboratory mice, we plan to evaluate the candidate JEV nucleic acid vaccine in pigs and nonhuman primates under experimental conditions. The pig is the natural host of JEV. It is the leading cause of stillbirth and abortion in sows in the epizootic area. Efficacy and safety testing of this candidate vaccine in pigs can be conducted in the epizootic area*

12. Human Subject Certification: Does this invention rely upon data involving human subjects as defined in and regulated under 45 CFR Part 46?

☒ No ☐ Yes → If "yes," please provide the Institutional Review Board (IRB) protocol approval number and date: _____ *or* explain fully below:

*before any human testing is performed.

PHS Employee Invention Report

Part I

First Inventor's Name: Chang, Gwong-Jen J.

2. A specific nucleic acid vaccine strategy has been developed for the prevention of infections caused by various flaviviruses. Japanese encephalitis virus (JEV) is the leading cause of human encephalitis in the Asian countries. We selected JEV as the test model for the following reasons: 1) the FDA-licensed JEV vaccine can serve as the vaccination control; 2) a common laboratory strain of outbred mice can be used to test the vaccine potency; 3) intraperitoneal or intranasal challenge of vaccinated mice can be used to assess the protective effect of vaccination; and 4) a new generation JE vaccine is needed for worldwide use to improve the existing mouse brain-derived inactivated vaccine. Three plasmids containing JEV PrM to E gene region were constructed that expressed PrM-E protein under the control of the cytomegalovirus immediate early protein promoter. A stable cell line transformed by p2-7 plasmid secreted JE virus-like particles into the culture media. This virus-like particle, containing processed M and E proteins, was identical to the purified JE virus in antigen-capture ELISA, western blot, and HI tests. We compared the potency of this nucleic acid vaccine with the FDA licensed inactivated human vaccine by intramuscular injection in three-day and three-week old mice. Seroconversion rates of 90 to 100% were observed in the nucleic acid vaccinated mice despite their age. Although the inactivated human vaccine induced 100% seroconversion in three-week old mice, none of the three- day old mice had measurable JEV specific antibody seven weeks postvaccination. The vaccinated female mice had plaque reduction neutralization antibody titer of 20 to 160 at nine weeks after immunization. The maternal antibody of the female mice provided 45 to 100% of passive protection of their progeny challenged at two days or two weeks with 1000 pfu of virulence JEV. Seven-week old adult mice that received JEV DNA vaccine at three days old showed 100% protection from 50,000 PAU of JEV challenge. JEV specific antibodies persisted in all mice that received one or two doses of nucleic acid vaccine eleven months after the initial immunization.

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6. (continuation)

Konishi, E., Pincus, S., Paoletti, E., Shope, R.E., Burrage, T., Mason, P.W. (1992). Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology. 188:714-20.

Phillpotts, R.J., Venugopal, K., Brooks, T. (1996) Immunization with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch Virol 141: 743-749.

8. Epidemics of flavivirus infections continue to be a major public health concern worldwide. More than two billion people currently reside in areas that are at risk of being infected with members of *flaviviridae,* including JEV in Asia, yellow fever virus (YFV) in Africa and Latin America, and four serotypes of dengue (DEN) virus in the tropic and subtropic regions of the world. A single JEV nucleic acid vaccine induced long lasting, protective immunity in adult or neonatal mice. Two to three-doses are recommended to use the existing mouse brain-derived inactivated or attenuated SA14-14-2 vaccines. Both vaccines are not recommended as a neonatal vaccine. We intend to apply the same strategy that has been tested in the JEV model to develop the nucleic acid vaccines for the four DEN serotype and YF viruses. Including nucleic acid vaccines for DEN viruses, YFV, and JEV in the World Health Organization's early childhood immunization program would create an immense commercial potential of worldwide markets.

13. First Inventor Information: *(Provide this information for each inventor who contributed to the essence of the invention. If more than one, use Page 4, "Information on Additional Inventors."*

Name	Degree	Social Security No. *(optional)*
Chang, Gwong-Jen J.	Ph.D.	521-31-4997
Position Title Research Microbiologist	**Office address** Centers for Disease Control and Prevention, PO Box 2087, Ft. Collins, CO 805	
Office Phone No. (970) 221-6497 / **FAX No.** (970) 221-6476	**Citizenship** ☒ U.S. ☐ Other:	

Home address 4237 Beaver Creek Drive, Fort Collins, CO 80526

Affiliation

☒ ICD *(specify ICD and applicable box below)* DVBID/NCID/CDC

☐ GS ☒ GM ☐ SES

☐ CO ☐ Visiting Fellow ☐ Visiting Associate

☐ Visiting Scientist ☐ Howard Hughes Fellow ☐ Guest Researcher

☐ Special Volunteer ☐ Other *(specify)*:

☐ Non-ICD Affiliation *(specify)*:

If more than one inventor, what specific contribution did you make to this work?

n/a

14. Inventors' Signatures

▶ This report is submitted pursuant to Executive Order 10096 and 10930 and/or Department Regulations. PHS employees have an obligation to report inventions they make while employed by PHS to OTT. Under E.O. 10096 and 367 CFR 501 the Government shall obtain the entire right, title, and interest in inventions: (i) made during working hours; or (ii) with Government facilities, equipment, materials, funds or information; or (iii) which bear a direct relationship or is made in consequence of the official duties of the inventor. If you are employed by PHS to conduct or perform research it is presumed that the invention was made under the foregoing circumstances. If this is not the case you must contact your Technology Development Coordinator (TDC) and provide the TDC with the details pertaining to this particular discovery or invention so that a determination of rights can be made.

Inventors' Signatures	Dates	Witnesses' Signatures	Dates
Gwong-Jen J Chang		R Cook Clo Jr	

Part II: To be completed by the Technology Development Coordinator.

15. Institute(s) or Agency(s) sponsoring this invention

CDC

16. Patent prosecution fees are to be charged to

CAN:	79210112		
ICD:			

Authorizing Official *(Typed)*	Signature	
Tech Transfer Asst.	Virginia McMillan	

▶ ***Send 3 copies of this form when completed to the OTT Patent Branch.***